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The purpose of this study is to understand why children with neurofibromatosis, type 1 (NF1) are at increased risk of developing myeloid leukemia and, in a broader sense, to utilize these cancer cells to generate new knowledge about the role of the NF1 gene *(NF1)* in growth control. Genetic and biochemical data from our laboratory provide formal proof that *NF1* acts as a tumor-suppressor gene in myeloid cells and regulates growth through its effects on the $p21^{ras}$ proteins. Boys with who inherit NF1 from their mothers are especially predisposed to leukemia, and children who receive chemotherapy and radiation for other cancers appear to be at increased risk of secondary leukemia. Finally, our data indicate that the leukemias of some children with no clinical evidence of NF1 carry *NF1* mutations.

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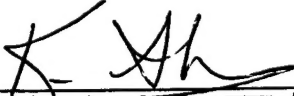
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Principal Investigator's Signature

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INTRODUCTION

Individuals with neurofibromatosis type 1 (NF1) are at increased risk of developing benign and malignant neoplasms including brain tumors, fibrosarcomas, and pheochromocytomas (1). All of these tumors arise in cells derived from the embryonic neural crest. Preleukemic myeloproliferative and myelodysplastic syndromes (MPS and MDS) are characterized by deregulated clonal proliferation of immature hematopoietic cells which show some myeloid differentiation *in vivo* (2). Juvenile chronic myelogenous leukemia (JCML) and bone marrow monosomy 7 syndrome (Mo 7) account for most cases of preleukemia in children and share many features including early age of onset, a predilection to affect boys, prominent enlargement of liver and spleen, leukocytosis, absence of the Philadelphia chromosome, evolution to acute myelogenous leukemia (AML), and a poor prognosis without bone marrow transplantation (2). Although preleukemia and AML are uncommon complications of NF1, children with NF1 are at markedly increased risk of developing these malignant myeloid disorders and comprise as many as 10% of *de novo* cases of preleukemia in the pediatric age group (3-5). These data, and a recent population-based study (6), indicate that there is a 200-500 fold increase in the incidence of leukemia in children with NF1.

The neurofibromatosis, type 1 gene *(NF1)* shares DNA sequence homology with eukaryotic GTPase activating proteins (GAPs) including human p120 GAP (also known as Ras-GAP) (7, 8). GAPs accelerate the conversion of the active GTP-bound form of p21^{ras} (Ras) to its inactive GDP-bound form by stimulating the intrinsic GTPase activity of Ras and therefore function, at least in part, as negative regulators of cell growth (7, 8). The biochemical properties of GAPs and the observation that activating point mutations of *RAS* occur frequently in human cancers suggested that *NF1* belongs to the tumor-suppressor class of recessive cancer genes. Structural analysis of malignant neural crest tumors and biochemical studies of fibrosarcoma cell lines derived from patients with NF1 support this model (9-13). In particular, fibrosarcoma cell lines contain reduced amounts of neurofibromin, a marked decrease in total cellular GAP activity and a high percentage of Ras in the active GTP-bound conformation (12, 13). While these data associate loss of neurofibromin activity with hyperactive Ras, elevated levels of Ras-GTP have not been demonstrated in fresh tumor samples from patients with NF1 and there is little information regarding the structural alterations in and biochemical activities of mutant *NF1* alleles retained in human malignancies. Furthermore, as all of the cell lines studied were of neural crest origin, these experiments provide no information about the role of neurofibromin in controlling the growth of hematopoietic cells. *RAS* mutations are not a feature of neural crest tumors and mutant Ras proteins promote differentiation in some neural crest cell lines (14, 15). These findings suggest that the biologic function of neurofibromin may differ between hematopoietic and neural crest cells. Characterizing how mutations identified in tumor cells alter the GAP activity of neurofibromin and its binding to Ras will provide important insights into how this protein normally controls the growth of hematopoietic and non-hematopoietic cells.

This research project had 3 objectives when it was approved in 1993: (1) to use molecular genetic methods to test the hypothesis that *NF1* functions as a tumor-suppressor gene in immature myeloid cells; (2) to determine if the Ras signaling pathway was deregulated in the leukemic cells of children with NF1; and, (3) to test the hypothesis that somatic alterations of *NF1* might contribute to the development of Mo 7 and JCML in children who do not show clinical stigmata of NF1. Supplemental funds were allocated from an award that was initially made to our collaborators at Onyx Pharmaceuticals in mid-1994 to facilitate additional biochemical analyses of patient materials. In addition to the experiments performed under these 3 objectives, we have carried out additional NF1-related research in human and mouse leukemias. The results of these studies are also presented briefly in this report.

DESIGN AND EXPERIMENTAL METHODS

<u>Collaborations</u>. Biochemical investigation of Ras-GTP levels and GAP activity in primary cancer cells was performed in collaboration with Drs. Gideon Bollag and Frank McCormick of Onyx Pharmaceuticals. In particular, all of these experiments were performed together by the Principal Investigator (Dr. Shannon) and Dr. Bollag. The novel method to measure GAP activity in permeabilized cells was developed in collaboration with Dr. Bollag for the specific purpose of accurately measuring the low levels of GAP activity present in primary human bone marrow cells. Other members of the NF research community have also facilitated this work. We have developed a productive collaboration with Dr. Tyler Jacks to investigate myelopoiesis and leukemogenesis in *Nf1* knockout mice, and Dr. Nancy Ratner provided us with antisera for some of the immunoblotting experiments described below.

<u>Patient samples</u>. We have investigated pathologic material from children with NF1 who developed malignant myeloid disorders. These samples were collected for clinical indications and were obtained from institutional and centralized cell banks. We extract parental DNA from peripheral blood cells: The experimental procedures have been approved by the Institutional Review Board of the University of California, San Francisco and are reviewed yearly. This project was also reviewed and approved by the Biology Research Committee of the Children's Cancer Group.

<u>DNA Extraction and Polymorphism Analysis.</u> Our methodology for DNA extraction, restriction endonuclease digestion, and Southern analysis have been described (16). During the course of this research, we adopted PCR-based methods for these experiments because these require less DNA, are less time-consuming, and because the markers are more informative. We routinely utilized 3 sequence polymorphisms within *NF1* and 1 marker located centromeric of the gene to screen for allelic loss. The intragenic markers were EVI-20 (16), an Alu repeat described by Xu et al. (17), and a complex repeat described by Andersen et .i. (18), and the flanking marker is UT-172 (16). In cases where these markers were not informative, we also

investigated two intragenic repeat polymorphisms described by Lazaro et al. (19). All of the polymorphic loci were scored by amplifying DNA segments that contain a variable number of short nucleotide repeats with flanking oligonucleotide primers. DNA samples were amplified in a DNA Thermocycle Machine (Perkin Elmer Cetus). PCR was performed in reaction mixtures that include 10 pM of respective 3' and 5' primers, 100 ng of target genomic DNA, 1 unit of Taq polymerase (AmpliTaq, Cetus Corp.), and 100 mM final concentrations of deoxynucleotides in a final reaction volume of 25 µL. ^{32}P deoxy-ATP was incorporated into the DNA fragments generated in the PCR procedure by adding 1 µL (10 µCi) of ^{32}P deoxy-ATP per 25 µL of the reaction mixtures and decreased the concentrations of "cold" deoxy-ATP to 50 mM. Labeled PCR products were separated on (6M urea, 8 percent polyacylamide) sequencing-type gels and run at 60-80 watts constant power for 2-4 hours. The gels were placed in Saran wrap and exposed to X-ray film for 1-36 hours at -70°C. The thermocycle parameters and experimental procedures for each primer pair were as described previously (16, 20). Investigating these leukemic samples for allelic loss was complicated by the fact that normal non-hematopoietic tissue from the same patients were not available to determine if their bone marrows showed loss of constitutional heterozygosity (LOH) in the *NF1* region. We therefore extract parental DNA when available and studied it in parallel with DNA from the patient bone marrows.

<u>Isolation of CD34+ Cells</u>. Frozen cells were thawed gradually and washed once in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal calf serum prewarmed to 37°C. Nucleated cells were labeled with 7.5 µM Hoechst 33342 (HO) at 37°C for 60 minutes, washed in Hank's balanced salt solution containing 0.02% azide, and incubated at 4°C for 45 minutes with fluorescein isothiocyanate-conjugated (FITC) anti-CD34 antibody (Becton Dickinson (BD), San Jose CA). Following antibody labeling, the cells were washed once with PBS. Dead cells were stained with 1 µg/mL propidium iodide (PI; Calbiochem, Santa Clara CA) for 5 minutes at 20°C. Flow cytometric analysis was performed using a FACStar PLUS (BD) equipped with 2 argon lasers tuned to 488 nm and 351-364 nm (ultraviolet wavelength). Forward light scatter, perpendicular light scatter, and fluorescence signals were measured for each cell. Cell doublets and aggregates were excluded using forward light scatter processing. HO and PI fluorescence emission (ultraviolet excitation) were collected through a 425 nm (± 50 nm) bandpass and a 620 nm long pass filter, respectively. PI^{dim}/HO-positive events were considered to be viable nucleated cells. FITC emission (488 nm excitation) was collected through a 530 nm (± 30 nm) bandpass filter. An isotype-matched antibody (Stimultest, BD) was used to define the immunofluoresence intensity above which cells were considered labeled. PI^{dim}/HO-positive CD34+ cells were sorted into a test tube containing RPMI medium and DNA was extracted as described previously (5). The small numbers of bone marrow mononuclear cells available from these young children precluded utilizing the cell sorter to purify additional hematopoietic subpopulations for DNA extraction and polymorphism analysis.

Single Strand Conformational Polymorphism Analysis of PCR Products. We initially utilized SSCP as a primary method for analyzing the *RAS* and *NF1* genes for mutations. SSCP proved robust for the *RAS* experiments because the mutations are concentrated in 2 small exons. However, SSCP proved unreliable and inefficient for studying the 59 exon *NF1* gene and we switched to a coupled in vitro transcription/translation (IVTT) assay that allowed us to scan the gene for mutations that resulted in protein truncation. Our methodology for SSCP has been described in detail in a previous progress report and in our published work (21, 22).

In Vitro Transcription/Translation Procedure. We used general experimental conditions and oligonucleotide primers that have been described previously to analyze *NF1* (23). Total cellular RNA (1-5 mg) was used as template for cDNA synthesis in a 25 µL reaction containing 1 µg random hexamers, 0.5 µg single stranded binding protein, 20u RNAsin, and 300u superscript II reverse transcriptase (Gibco/BRL). This reaction is incubated at 37°C for 1 hour. The cDNA is amplified in duplicate, using 1 µL and 3 µL template volumes with 5 overlapping primer pairs which amplify the entire *NF1* coding sequence in 5 segments of approximately 2 kb each (23). The 5' primer contains a T7 promoter sequence as well as a translation initiation site. A PCR mix containing 1X buffer (50 mM KCl, 10 mM Tris, pH 8.3, 2 mM $MgCl_2$), 0.8 mM dNTP's, and 10 pM of each primer is added to the cDNA. A wax gem is added, the 40 µL reaction heated to 80°C for 5 minutes then cooled to room temperature, and 10 mL of top mixture containing 1X buffer, 0.25 µg single stranded binding protein, and 2.5u Taq polymerase is added. PCR amplification is performed in a Perkin-Elmer 9600 thermocycle machine using the following conditions: initial denaturation at 95°C for 1 minute followed by 40 cycles of PCR consisting of 95°C for 30 sec, 62.5°C for 30 sec, 72°C for 90 sec , and 10 minute elongation at 72°C. A 5 µL aliquot of PCR product is electrophoresed on a 1% mini-agarose gel to check amplification. In the translation step, 3 µL of amplified cDNA and 10 µCi of ^{35}S labeled methionine are added to 0.25 µL T7 polymerase, 4 µL rabbit reticulocyte lysate, 0.16 µL amino acid mix (minus methionine), 0.16 µL RNAsin, 0.33 µL TNT buffer (Promega T7 TNT kit) and incubated at 30°C for 1 hour. A 3 µL aliquot of this reaction is added to 25 µL of reducing buffer (10% glycerol, 5% 2-mercaptoethanol, 2% SDS, 0.1% bromphenol blue) and boiled for 5 minutes. Protein electrophoresis is then performed on a 12.5% SDS/polyacrylamide gel at 30 mA for 3 hours in 1X TGS running buffer. The gel is fixed in 30% methanol/10% acetic acid, and treated with Entensify (Dupont) prior to autoradiography. Protein size is mapped by comparing migration of abnormal bands to a standard of known molecular weight.

Cloning and sequencing strategy. We clone and sequence PCR products that migrate abnormally in SSCP gels or give rise to truncated proteins using the CloneAmp vector system (Gibco BRL). We have successfully applied this method to clone and sequence *RAS* mutations identified by SSCP as described elsewhere (21, 22). We modified the primers described by Heim et al. (23) to amplify *NF1* to incorporate the sequence 5'CUACUACUACUA3' at the 5' end of the sense primer and

5'CAUCAUCAUCAU3' at the 5' end of the antisense primer. PCR products are digested and 10 ng is then annealed to the precut vector in the presence of uracil DNA glycosylase. The annealed products are then transformed into DH5α-competent cells, streaked, and grown overnight on ampicillin plates. Before sequencing products that yield truncated polypeptides, we perform second round of IVTT on bacterial lysates to differentiate clones containing mutant from those containing wild type sequences. In this procedure, colonies are picked, grown up in 5 mL of L broth, and 5 μL of bacterial culture is added to 50 μL of distilled water. The mixture is boiled for 10 minutes, insoluble material is removed by centrifugation, and 5 μL of lysate is used as template for PCR amplification followed by IVTT. Colonies containing plasmid DNA which gives rise to truncated polypeptides are selected and DNA extracted by standard methods. A polyethylene glycol precipitation is performed to purify the DNA further and sequencing is performed using Sequenase, version 2.0 (USB).

Immunoprecipitation of Neurofibromin from Cell Lysates and Western Blotting. Sf9 insect or mammalian cells (100 μL) are lysed in 400 mL of buffer consisting of 50 mM Tris, 1% NP-40, 0.5% DOC, 0.1% SDS, 150 mM NaCl and protease inhibitors, vortexed vigorously for 2 minutes, and placed on ice for 10 minutes. The cells are then spun at top speed in a benchtop Eppendorf centrifuge at 4°C for 15 minutes and 100 mL aliquots are transferred to fresh tubes. Antisera (3-5 μL) are added and the samples are incubated on ice for 1-2 hours. We then add 50 μL of Proteinase A sepharose bead solution (prepared in PBS) and mix at 4°C for 30 minutes. The beads are spun at 4°C and washed 4 times in 1 mL of lysis buffer. The pellet is resuspended in 30 μL of sample buffer, placed at 95°C for 5 minutes to release protein-antibody complexes from the beads, and spun for 10 seconds to precipitate the beads. We load 10 μL aliquots per lane on gradient acrylamide gels (4 to 20%) run at 200 volts for 45 minutes in Tris/glycine/SDS running buffer. Protein is transferred onto nitrocellulose filters for 3 hours at 300 mA in Tris/glycine/SDS buffer containing 20% methanol. The filters are blocked in low detergent Blotto for 1 hour to overnight. The primary antibody is added to Blotto and incubated with the filters from 1.5 hours to overnight. The filters are washed 3 times in 1X PBS/0.05% Tween 20 (TPBS) for 10 minutes. Secondary antibody is then added to 1X TPBS containing 1% BSA and the filters are incubated an additional 1-2 hours. The filters are washed 3 times in TPBS as above, developed with ECL (Amersham), and exposed to autoradiography.

Measurement of Ras-GTP Levels. We modified the method of Downward et al. (24) to measure the relative amounts of GDP and GTP bound to Ras in living cells and described this in detail in our previous progress report and in a subsequent article (25).

Determination of GAP Activity. The phosphate release GAP assay has been described elsewhere (26). Briefly, cells are resuspended at 10^6 cells per tube in 1.5 mL Eppendorf microcentrifuge tubes and pelleted. After aspirating the medium, the

cells are either assayed immediately or frozen in a dry ice/ethanol bath for storage at -80°C. To measure total GAP activity, the cell pellet is lysed in a solution consisting of 0.1% NP-40; 20 mM Tris, pH 7.5; 2 nM $MgCl_2$, and 2 mM DTT. The GAP activity of a lysate is assayed by measuring the amount of phosphate released from recombinant *NRAS* loaded <u>in vitro</u> with $(\gamma$-$^{32}P)GTP$ during an 8 minute reaction performed at room temperature (22-25°C). After incubation, the reaction is quenched with acid, the free phosphate is chelated by amonium molybdate, and the phosphomolybdate is extracted with isobutanol: toluene (1:1). The upper (organic) layer is removed and counted to quantify the amount of $(^{32}P)GTP$ hydrolyzed to GDP and free ^{32}P. Samples are run in duplicate or triplicate.

Neurofibromin, p120 GAP, and, probably, one or more uncharacterized proteins contribute to total cellular GAP activity. The GAP activity of neurofibromin is very sensitive to inhibition at concentrations of dodecyl maltoside (DDM) that do not significantly alter the activity of p120 GAP (26). This biochemical difference between *NF1* GAP and p120 GAP has been exploited to determine the fraction of total cellular GAP activity attributable to each protein.

We found very low GAP activities in normal and leukemic bone marrow cells using the standard phosphate release assay (25). For this reason, we developed a new *in vivo* assay of GAP activity. In this method, we label recombinant, processed *KRAS* with $\alpha^{32}P$-GTP as above. A total of 2×10^6 marrow mononculear cells are washed and suspended in 1 mL of a permeabilization buffer consisting of 6.25 mM $MgCl_2$, 1.25 mM ATP, 1.25 mM Pipes, pH 7.4, 150 mM KCl, 1.25 mM EGTA, 0.75 mM $CaCl_2$, and 37.5 mM NaCl. Streptolysin O (0.2 mL) and recombinant *KRAS*- $\alpha^{32}P$-GTP is added to each tube and to a control tube containing permeabilization buffer alone. Aliquots of 5×10^5 cells are removed after 2, 5, 10, and 30 minutes, lysed, and immunoprepcipated with monoclonal antibody Y13-259. The ratio of Ras-GTP to Ras-GDP at each time point is determined using the same procedure for ^{32}P orthophosphate loading.

RESULTS

Objective 1. *To Test the Hypothesis that NF1 Functions as a Tumor-Suppressor Gene in Myeloid Leukemia in Children with NF1*

According to the Knudson model, the single normal *NF1* allele will be inactivated in the bone marrows of children with NF1 during leukemogenesis if this gene functions as a tumor-suppressor in immature myeloid cells (27). We have investigated marrow samples from 23 children and their parents for allelic loss at *NF1* with a series of polymorphic markers including 11 patients reported previously (16) and 12 unpublished cases (28, 29). Nine leukemias showed loss of a parental allele. Eight of these marrows were obtained from children with familial NF1 and the *NF1* allele inherited from the unaffected parent was deleted in all cases. We studied lymphoblastoid cell lines transformed with Epstein-Barr virus (EBV) from 8

patients including 4 with allelic losses at *NF1*. We also isolated CD34+ cells from the frozen bone marrow mononuclear cells of 3 of these 4 patients, and plucked erythroblasts from BFU-E colonies grown from 1 child. As shown in Figure 1, the EBV lines of 3 children with JCML retained both *NF1* alleles while the CD34+ cells consistently demonstrated loss of constitutional heterozygosity (LOH). Taken together, these data support the hypothesis that *NF1* functions as a tumor-suppressor gene in hematopoietic cells and indicate that leukemic transformation in JCML occurs at the level of an immature progenitor that is committed to myeloid/erythroid differentiation. In contrast, we recently studied a 10 month old child with an unusual myeloproliferative disorder who showed loss of the normal parental *NF1* allele in both EBV-transformed cells and bone marrow (data not shown). This result indicates that inactivation of *NF1* may occur in a more primitive precursor in some patients.
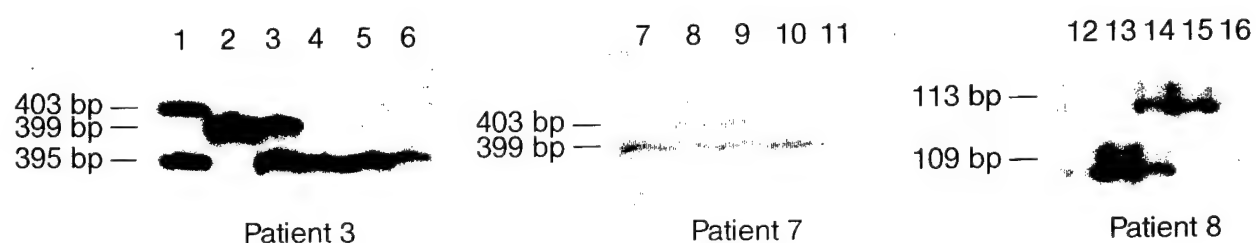

Figure 1. Analysis of bone marrow and EBV cell line DNA from 3 children with NF1 and JCML, and DNA extracted from the blood of their parents. The mother is affected with NF1 in all 3 families. Lanes 1, 7 and 12 show maternal DNA, lanes 2, 8 and 13 show paternal DNA, lanes 3, 9 and 14 show EBV line DNA and 4, 10 and 15 show DNA extracted from leukemic bone marrow. Lanes 5, 11 and 16 represent DNA isolated from CD34+ cells in patient marrow and lane 6 shows analysis of burst forming unit-erythroid (BFU-E) colonies from one child. In all 3 cases, polymorphic *NF1* markers demonstrate that the EBV lines have retained both parental alleles whereas the affected bone marrow samples have deleted the normal paternal *NF1* allele.

NF1 is a challenging target for mutation analysis because the gene spans 59 exons; a combination of SSCP and Southern analysis detected only 20% of the causative mutations in patients who fulfill NIH clinical criteria for NF1 (30). We therefore adapted an IVTT assay to screen leukemic samples for *NF1* mutations that lead to premature termination of protein translation. This technique was developed to screen for mutations in the *APC* gene and is theoretically appealing for investigating candidate tumor-suppressor genes because tumorigenesis is associated with loss of function (31). We have performed IVTT on EBV cell lines and/or bone

marrow samples from 18 patients that were available for RNA extraction and detected truncated proteins in 8 of them. Representative data are shown in Figure 2.
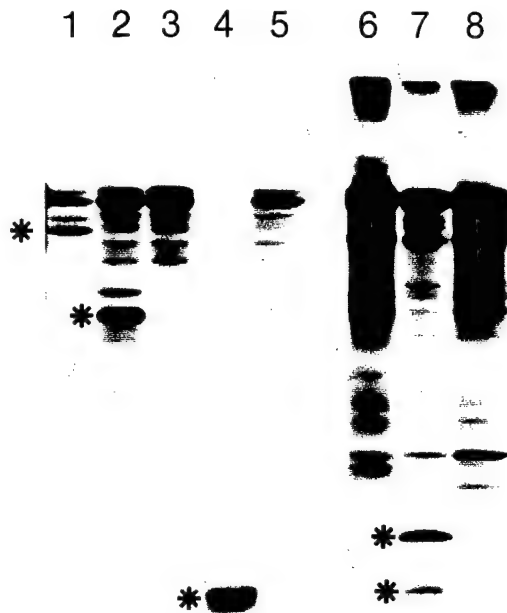

Figure 2. IVTT of 5 EBV cell lines (lanes 1-5) and 3 bone marrows (lanes 6-8) from children with NF1 and myeloid disorders. Lanes 1-5 show polypeptides synthesized from cDNAs amplified using segment 3 primers (exons 19b-29). Lanes 6-8 show polypeptides from segment 2 templates (exons 10b-21). Truncated polypeptides (lanes 1, 2, 4 and 7) are denoted by an asterisk. The sample in lane 4 shows complete absence of normal protein bands consistent with loss of the wild type *NF1* allele in both patient bone marrow and in the EBV line (data not shown). We have confirmed *NF1* mutations in each of the samples that gave abnormal results by IVTT.

Mutations have been confirmed by sequencing cloned cDNA in 7 children to date and are these are summarized in Table 1. Of the 7 fully characterized abnormalities, 2 were nonsense and 5 were frameshift mutations. Each of the *NF1* mutations identified by IVTT was also present in genomic DNA extracted from the patient's leukemic bone marrow. The bone marrows of 5 patients with truncating *NF1* mutations also showed LOH at *NF1*. Thus, these leukemias show inactivation of both *NF1* alleles. Two of the 7 marrows that carried truncating *NF1* mutations were obtained from children with sporadic NF1 (Table 1). These mutations have been documented previously in 3 unrelated patients with NF1 (23), none of whom developed leukemia. In the 5 patients with familial NF1, genomic DNA from each of the affected parents harbored the same mutation as the child. Figure 3 shows DNA amplified from a child with JCML and his parents, using oligonucleotide primers flanking exon 28 which contains a 4 nucleotide deletion, demonstrating both LOH and the presence of the mutation in the germline.
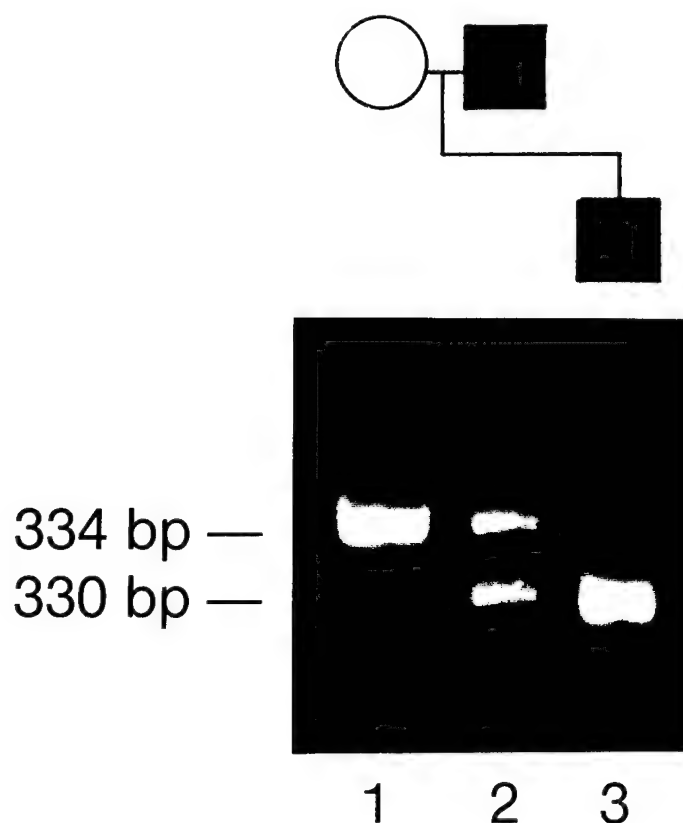
Figure 3. Analysis of patient 9 (Table 1) and his parents. Exon 28 of *NF1* was amplified from genomic DNA from parental blood and the leukemic marrow. A shorter PCR product corresponding to the 4 bp deletion is seen in the father (who has NF1) and in the child's leukemia. There is no normal fragment seen in the leukemic bone marrow which also showed LOH with polymorphic markers.

We wished to determine if the truncated proteins synthesized *in vitro* would be stably expressed *in vivo*. This is important since in at least 3 of the 7 cases the GAP domain of neurofibromin, encoded by exons 20-27b, may potentially be translated and the truncated protein might therefore retain some function. We performed immunoblotting experiments on the EBV cell lines shown in Figure 2 using an antibody against the amino terminus of neurofibromin (kindly provided by Dr. Nancy Ratner). We did not detect expression of these truncated proteins and observed complete absence of neurofibromin in the EBV line with LOH (Figure 4).
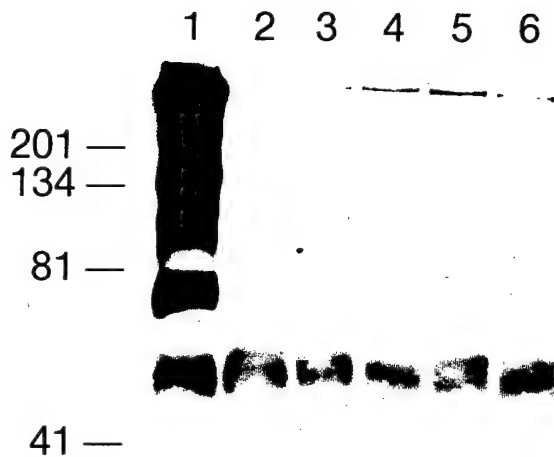
Figure 4. The proteins shown on this Western blot were expressed by EBV lines derived from shown in Figure 2. Immunoprecipitation was performed using an antibody to the amino terminus of neurofibromin, and the filter was probed with a secondary antibody, also within the amino-terminal domain. Lanes 3 and 4 are from EBV lines that showed a normal peptide pattern on the protein truncation assay, lanes 5 and 6 showed both normal and truncated peptides and lane 2 showed only a truncated peptide *in vitro*. Here only normal sized peptide is apparent in lanes 3 through 6 and there is no demonstrable protein in lane 2, where the EBV line has deleted the normal allele.

When the patients studied in our laboratory are combined with cases of *de novo* malignant myeloid disorders in children with NF1 reported by other investigators, skewing is evident with respect to the sex of the parent transmitting NF1 as well as the sex of the affected child (28). These data are summarized in Table 2. The ratios of maternal to paternal transmission and of boys to girls with myeloid disorders were 2.3:1 and 4.5:1, respectively. The most pronounced difference were seen between boys who inherited NF1 from their mothers (18 cases) and girls who inherited it from their fathers (1 case). By chi square analysis, there was a significantly greater proportion of mothers transmitting NF1 to children who developed leukemia ($p < .02$) and a significantly greater proportion of affected boys with familial NF1 ($p < .01$).

Our data showing that *NF1* acts as a tumor-suppressor in immature myeloid cells are consistent with the proposal that neurofibromin negatively regulates the growth of myeloid cells by acting as a GAP for Ras. This model predicts that acquired *RAS* mutations will be restricted to children who do not have NF1 because an oncogenic Ras protein would be biochemically redundant in cells with defective neurofibromin GAP activity. We and our collaborators utilized SSCP, oligonucleotide hybridization, an allele-specific restriction endonuclease assay, and DNA sequencing to test this hypothesis in 91 children with malignant myeloid disorders. We detected activating *RAS* mutations in 15 of 72 cases of preleukemia in children without NF1 and in 0 of 19 patients with NF1 ($p = 0.02$ by chi square) (21, 32). Since publishing these findings, we investigated 6 more leukemic samples from children with NF1 for *RAS* mutations and found none (data not shown). These results provide strong genetic evidence that neurofibromin regulates myelopoiesis through its effects on Ras.

Objective 2. *To determine if the Ras signaling pathway is deregulated in the leukemic cells of children with NF1*

A number of methods exist to address the role of neurofibromin in regulating Ras proteins including: (1) determining GAP activity in cell lysates; (2) overnight labeling of intact cells with ^{32}P orthophosphate to directly measure the relative amounts of GTP and GDP bound to Ras; and (3) assaying the activation state of proteins downstream of Ras such as MAP kinase. We have utilized all of these techniques to investigate primary human bone marrow cells in collaboration with Drs. Gideon Bollag and Frank McCormick (25). The hypothesis that *NF1* functions as a tumor-suppressor by negatively regulating Ras predicted that GAP activity would be decreased, that Ras GTP-levels would be increased, and that MAP kinase would be activated in the bone marrows of children with NF1 who developed leukemia.

We first compared GAP activity in lysates prepared from normal bone marrows with lysates from the leukemic cells of children with and without NF1. Recombinant Ras was loaded with γ-^{32}P GTP and then incubated with lysates prepared from fresh or cryopreserved bone marrow mononuclear cells. The detergent dodecyl maltoside (DDM), which selectively inhibits the GAP activity of neurofibromin, was added to some of the reactions to distinguish "neurofibromin (NF1-like) GAP activity" from "p120-GAP activity" (26). As shown in Figure 5, NF1-like GAP activities were significantly lower in lysates prepared from NF1-associated leukemias than in either normal marrows (p = 0.01 by Mann Whitney U) or non-NF1 leukemias (p = 0.03). There were no significant differences in p120-like GAP activities (25).
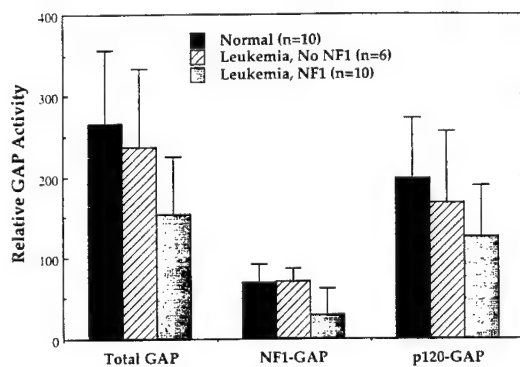
Figure 5. Ras GAP activities in bone marrow cells from unaffected individuals, patients with leukemia without NF1, and patients with leukemia and NF1. GTP hydrolysis was assayed using cell lysates in the absence (total GAP activity) or presence (p120-like GAP activity) of dodecyl maltoside, a selective inhibitor of neurofibromin (NF1-like GAP activity). Data are normalized to Ras-GTP hydrolysis in the absence of lysate, and error bars indicate the standard deviations (25).

Because neurofibromin accounts for only 20-30% of total GAP activity in bone marrow cells (Figure 5), the NF1 leukemias retain most of their GAP activity despite the marked reduction in NF-like GAP activity. We therefore asked if p120 GAP might be activated during the lysis procedure. In order to address this question, we developed an assay in which we introduced recombinant Ras preloaded with α-^{32}P GTP into permeabilized bone marrow mononuclear cells, lysed the cells at defined time points, and measured the relative amounts of labeled GTP and GDP eluted from Ras immunoprecipitates. The rates of GTP-to-GDP hydrolysis on Ras were similar over time in normal bone marrow cells, non-NF1 leukemias, and NF1 leukemias (25). We conclude that the leukemias of children with NF1 show a marked reduction in the NF1-like component of GAP activity yet retain normal (or near-normal) levels of total GAP activity.

We first attempted to measure the percentages of Ras-GTP and Ras-GDP in cryopreserved JCML and normal bone marrow cells. However, thawed cells equilibrated the phosphate level poorly and gave inconsistent results which correlated with viability. We therefore performed ^{32}P orthophosphate labeling experiments in parallel on freshly-isolated mononuclear cells from 4 children with NF1 and 4 normal bone marrows under a variety of conditions. The 4 NF1 patients included 2 with JCML, 1 with acute myelomonocytic leukemia, and 1 who developed myelodysplastic syndrome with monosomy 7 a few months after completing treatment for astrocytoma. The percentage of Ras-GTP in these patient bone marrows were uniformly higher by about 10% than the corresponding levels in normal cells under a variety of culture conditions. These data demonstrate that primary leukemic cells from children with NF1 demonstrate moderate, but consistent, elevations in the percentage of Ras-GTP (25).

We also tested the hypothesis that the activity of mitogen activated protein (MAP) kinase (a downstream effector of Ras signaling) would be elevated in bone marrow cells from children with NF1 and malignant myeloid disorders. To address this question, we used an antibody against MAP kinase to immunoprecipitate this protein from bone marrow lysates. Kinase activity was assayed by measuring phosphorylation of a myelin basic protein substrate. Uninduced lysates from 5 of 8 NF1-associated leukemias showed MAP kinase activities above the range seen in 5 normal bone marrows (Figure 6).
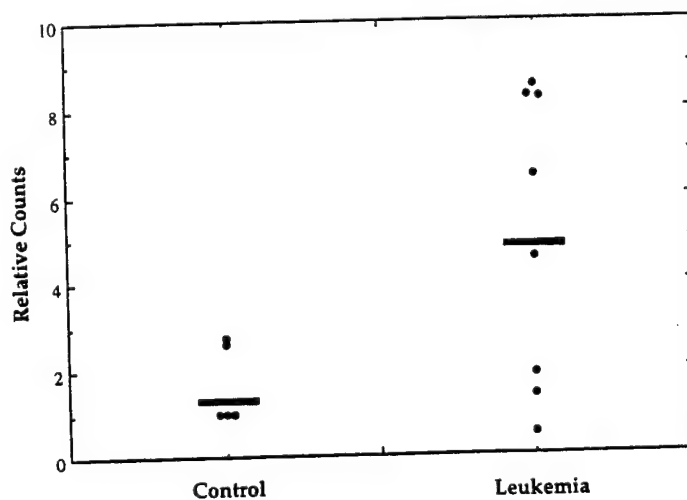

Figure 6. Relative MAP kinase activities in normal individuals compared with children with NF1 and leukemia. Values are expressed as counts relative to the lowest normal control. Data from individual analyses are indicated by closed circles, while the average values are indicated by horizontal bars.

In conclusion, we detected 3 distinct biochemical abnormalities in patient leukemias: a reduction in NF1-like GAP activity, elevated levels of Ras-GTP, and MAP kinase activation. These findings are consistent with our genetic data and strongly support a model whereby neurofibromin restrains the growth of immature myeloid cells by negatively regulating the Ras pathway.

We have also had the opportunity to characterize a novel mutant *RAS* allele that we detected during our study that ascertained the incidence of *RAS* mutations in leukemic bone marrows from children with and without NF1 (21). SSCP analysis of *KRAS* exon 1 fragments amplified from the marrow DNA of a 4 year old boy with acute myelogenous leukemia (AML) and monosomy 7 who did not have NF1 revealed two abnormal fragments that migrated more slowly than the normal bands (Figure 7). The intensities of the normal and abnormal fragments were equivalent (Figure 7). Surprisingly, allele-specific oligonucleotide analysis showed no mutations at codons 12 or 13, and subsequent sequence analysis of cloned PCR products obtained after amplifying the complete coding region of exon 1 demonstrated an in-frame 3 nucleotide insertion (GGA) between codons 10 and 11 in multiple clones We designated this mutant K-Ras[10G11] to indicate the insertion of a glycine residue between amino acids 10 and 11.
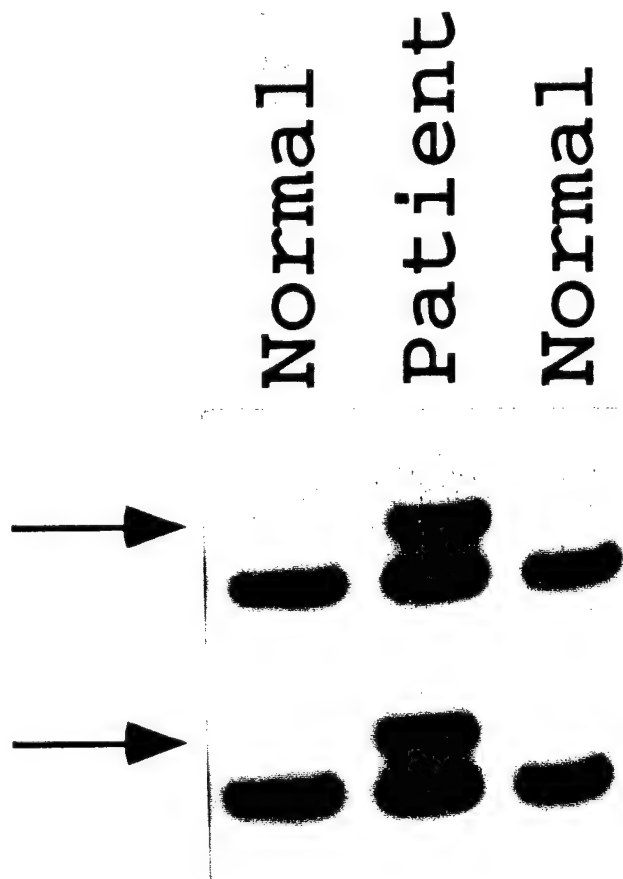

Figure 7. Detection of an insertion in exon 1 of *KRAS* in the bone marrow of a child with AML. The samples in lanes 1 and 3 show a normal migration pattern. These bands are also seen in the patient sample (Lane 2) along with 2 abnormal fragments that are visible above the normal products.

K-Ras[10G11] efficiently transformed NIH 3T3 cells in a focus forming assay. In addition, biochemical analysis was carried out to compare K-Ras[10G11] to a common activating point mutation (K-Ras[Asp 12]) and to wild-type Ras (K-Ras[WT]). As shown in Figure 8, the percentage of GTP-bound K-Ras[10G11] was remarkably elevated (93.5%) versus either K-Ras[Asp 12] (45.7%) or K-Ras[WT] (7.0%) in transfected COS cells. K-Ras[10G11] showed a profound reduction in intrinsic GTP hydrolysis and the protein was resistant to both p120 GAP and neurofibromin. This novel mutation underscores the importance of deregulated Ras signaling in myeloid leukemogenesis. Like other mutant Ras proteins, the elevated level of GTP-bound K-Ras[10G11] in intact cells is likely to result, at least in part, from the inability of either p120GAP and/or neurofibromin to accelerate GTP hydrolysis.

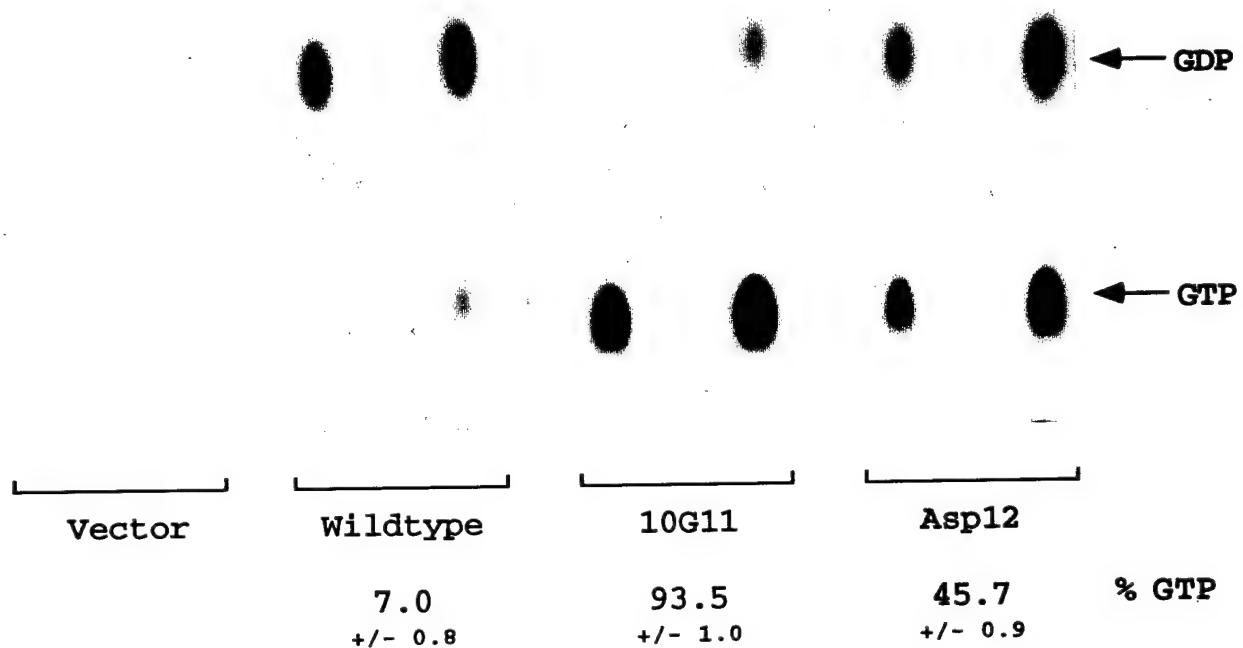**Figure 8.** Autoradiographs of nucleotides bound to K-Ras[WT], K-Ras[10G11], and K-Ras[Asp 12] expressed in COS cells. Nucleotides eluted from immunoprecipitated Ras proteins from cells transfected with empty vector (lanes 1, 2), K-Ras[WT] (lanes 3, 4), K-Ras[10G11] (lanes 5, 6), and K-Ras[Asp 12] (lanes 7, 8) were separated by thin-layer chromatography and the GTP to GDP ratios were measured in an Ambis scanner.

Objective 3. _To investigate bone marrow samples from children with Mo 7 and JCML who do not show clinical stigmata of NF1 for NF1 alterations_

We initially investigated DNA extracted from 27 Mo 7 bone marrows for LOH at _NF1_ but did not detect allelic loss in any of them (16). Because JCML is the most common leukemia seen in children with NF1, we next analyzed bone marrow specimens from 21 patients. We were especially interested in studying marrows from children < 2 years old because the clinical stigmata of NF1 may be not be apparent in young children. We have detected LOH at _NF1_ in a single child. We also extracted RNA from these marrows for IVTT. An abnormal peptide fragment was detected from the bone marrow sample which showed LOH and 2 other cases without LOH. The bone marrow that showed both LOH and an abnormal peptide fragment by IVTT was obtained from a 3 1/2 month old baby with JCML, a negative family history of NF1, and no stigmata of the disease. We have defined a splice donor mutation (6365 + 18 A to G) in this case and found a G to A substitution (Trp to Stop) at nucleotide 4614 of _NF1_ mutation in another patient sample. We are still characterizing the third mutation. It will be of interest to determine if these _NF1_ mutations are restricted to the leukemic clone or exist in the germline.

Other NF1-Related Research

This section briefly describes other NF1-related research that was not included in the Objectives, but represented natural extensions into new areas. All of the murine experiments and most of the studies of human leukemia were funded by other intramural and extramural sources. However, limited support for the studies of chromosome 7 loss, secondary leukemia, and brain tumors was provided by this Army award.

Loss of Chromosome 7 Alleles in Children with JCML. Although children with NF1 are at markedly increased of developing leukemia relative to the general population, the overall incidence is low. This suggests that other genetic events cooperative with inactivation of _NF1_ in leukemogenesis. The bone marrows of many children with NF1 show chromosome 7 deletions. We therefore asked if the bone marrows of some patients with preleukemic disorders might show LOH on chromosome 7 by genetic mechanisms that do not result in cytogenetically-detectable deletions. We used a series of polymorphic markers from chromosome 7 to address this question in 19 children with JCML, including 5 with NF1. We found no evidence for allelic loss, a result that suggests that JCML and Mo 7 are distinct disorders that share one or more molecular alterations in common (33).

Secondary Leukemia in Children with NF1. An interesting and concerning recent finding has been that 5 children with NF1 have developed malignant myeloid disorders with monosomy 7 after multi-modal therapy for other cancers (29). Our collaborator Dr. Maris reviewed the Tumor Registry at the Children's Hospital of

Philadelphia and found an incidence of second cancer of 10% in children with NF1. Of 8 patients with embroynal cancers, 6 developed a second cancer (29). This is perhaps due to the fact that these young children typically receive aggressive multi-modal therapy that includes both chemotherapy and radiation. As expected, we have not detected *RAS* mutations in any of these marrows and none have shown LOH at *NF1*. These cases indicate that mutagenic therapy can interact with the genetic predisposition to myeloid leukemia that exists in patients with NF1 and therefore have important therapeutic implications. Additional molecular investigation of these patients is in progress.

<u>Molecular Analysis at NF1 in Astrocytic Brain Tumors</u>. A summer student in the lab used the polymorphic *NF1* markers to investigate of sporadic astrocytic brain tumors for LOH. The rationale for these experiments was that astrocytomas are common in individuals with NF1 and it was therefore possible that somatic alterations of *NF1* contributed to the development of sporadic tumors. We detected LOH in a single recurrent astrocytoma (20). These data are consistent with other reports which suggest that *NF1* mutations play a limited roles in neural crest rumors that develop in patients without NF1.

<u>Hematopoietic Colony Growth in Murine Fetal Liver Cells.</u> A hallmark of JCML cells is selective *in vitro* hypersensitivity to the hematopoietic growth factor granulocyte-macrophage colony stimulating factor (GM-CSF) (34). Because leukemic cells often contain multiple genetic alterations, the relationship between inactivation of *NF1* and aberrant growth in response to GM-CSF was uncertain. We therefore addressed how loss of *Nf1* function affects hematopoietic progenitor growth in murine fetal liver cells in collaboration with Dr. Jacks and Dr. Wade Clapp of Indiana University. Cells harvested from day 12.5 *Nf1* embryos were cultured in methylcellulose medium and a range of recombinant murine GM-CSF concentrations. Control cultures were established in the presence of murine interleukin 3 (IL-3). Cultures of homozygous mutant *(Nf1-/-)* cells yielded more myeloid colonies at low GM-CSF concentrations than cells from wild-type *(Nf1+/+)* or heterozygous *(Nf1+/-)* litter mates. In contrast, the dose-response relationship for myeloid colony formation was similar for *Nf1+/+, Nf1+/-,* and *Nf1-/-* cells in the presence of IL-3. These experiments show that disruption of *Nf1* confers an aberrant pattern of hematopoietic progenitor colony growth that is remarkably similar to that seen in JCML. The results were presented in the paper that described the biochemical abnormalities in leukemic cells isolated from children with NF1 (25).

<u>Murine Transplant Model.</u> The finding of GM-CSF hypersensitivity in *Nf1-/-* fetal liver cells suggested that these cells could be used to develop a murine model of myeloid leukemia. We therefore mated *Nf1+/-* animals, harvested the embryos at day 12.5 - 13.5, and injected hematopoietic cells isolated from fetal livers into irradiated syngeneic mice. The recipients predictably developed a JCML-like disorder with markedly increased white blood cell counts and infiltration of liver and spleen with myeloid cells. Largaespada et al. (35) independently obtained similar results in a different strain of *Nf1* knockout mice. We are using this model

to test experimental therapeutics and to further characterize the role of neurofibromin in regulating hematopoietic proliferation.

CONCLUSIONS

The genetic studies carried out under Objective 1 demonstrate that the normal *NF1* allele is frequently lost from the bone marrows of children with NF1; that homozygous inactivation of both alleles exist in some samples; and that truncated neurofibromin peptides are not stably expressed *in vivo*. These results provide a formal genetic proof of the hypothesis that *NF1* functions as a tumor-suppressor gene in the bone marrows of children who develop leukemia. Our data are in agreement with the observation that a number of neural crest tumors from patients with NF1 have shown LOH at *NF1* including pheochromocytomas (10), neurofibromas (36), and fibrosarcomas (9, 37). However, rigorous proof that *NF1* functions as a tumor-suppressor requires demonstrating inactivation of both alleles in spontaneously arising human tumors. Tumor-specific deletions of both *NF1* alleles have previously been shown in a single patient with NF1 who developed a neurofibrosarcoma (11). We have now conclusively shown inactivation of both *NF1* alleles (one by LOH and the other by gene mutation) in 5 leukemias from unrelated children. We speculate that both *NF1* alleles were inactivated in the leukemic cells of the other patients by mutations that did not result in detectable truncations of neurofibromin. Although we did not detect all of the *NF1* mutations in our patient samples, IVTT proved to be an efficient method to screen the large *NF1* coding region.

The absence of activating *RAS* mutations in the bone marrows of children with NF1 provide genetic evidence that the tumor-suppressor function of *NF1* is mediated through its effect on Ras. This hypothesis is strongly supported by the biochemical data obtained in the experiments performed under Objective 2 in which we foundd a significant reduction in NF1-like GAP activity, an increased percentage of Ras-GTP, and evidence for MAP kinase activation in primary bone marrow cells from children with NF1 who developed leukemia. We also developed a new method for measuring GAP activity in permeabilized cells that should prove useful to other investigators in the Ras signaling field. We have examined JCML and Mo 7 bone marrow samples from children without NF1 for alterations in the gene under Objective 3 and have shown that these occur in some patients.

The frequency of myeloid leukemia in children with germline mutations of *NF1* is much lower that the incidence of childhood cancer in patients with inherited alterations of the tumor-suppressor genes *P53*, *WT1* and *RB1* (27). One potential explanation of these data was that certain *NF1* alleles might strongly predispose to leukemia, perhaps by a dominant negative mechanism. Our data argue strongly against such a genotype-phenotype correlation because the mutations we have detected: (1) do not appear to encode stable peptides; (2) have been detected previously in individuals with NF1 without leukemia; and, (3) were not seen in families with a history of multiple children affected with leukemia. These data have important clinical implications and we have been contacted by a number of

families who asked about the risk that leukemia might develop in another child.

The low incidence of myeloid leukemia in children with NF1 suggests that inactivation of *NF1* cooperates with other inherited and acquired genetic factors. The striking finding of that most patients with myeloid leukemia are boys who inherited NF1 from their mothers is unexplained but implicates epigenetic factors in leukemogenesis. Our data showing that the mutant *NF1* alleles retained in the bone marrows of affected children could be inherited from either parent provides evidence that *NF1* is not imprinted in hematopoietic cells. Approximately 25% of children with NF1 who develop malignant myeloid disorders also show bone marrow monosomy 7 (5, 16). Taken together, the existing data suggest a multistep model in which loss of *NF1* function cooperates with epigenetic factors and with chromosome 7 loss and with other somatic mutations in leukemogenesis. The development of leukemia in children with NF1 offers an attractive system for further studies of the more general process of multistep tumorigenesis.

Our biochemical data are the first to directly examine the effects of loss of *NF1* function on cellular GAP activity and Ras-GTP levels in primary human cancer cells. The results of this analysis are provocative. In contrast to fibrosarcoma cell lines derived from patients with NF1 (12, 13), primary leukemic cells retain a near-normal level of total GAP activity with a specific reduction in the NF1-like component and a moderate elevation in Ras-GTP levels. These data are not unexpected as these leukemias retain p120 GAP. It is possible that the biochemical consequences of loss of *NF1* are different in different cellular contexts. An alternative explanation for the discrepancy between primary leukemic cells and fibrosarcoma cell lines from NF1 patients with respect to Ras-GTP levels is that the elevated values seen in fibrosarcoma cell lines are a secondary phenomenon that arises during out-growth in culture. Despite these differences, it is important to emphasize that biochemical data from both primary leukemias and fibrosarcoma cell lines support the proposition that Ras is deregulated in neurofibromin-deficient cells of the hematopoietic and neural crest lineages.

Our data showing that both *NF1* alleles are inactivated during tumorigenesis leading to deregulated Ras signaling have important implications for treating the complications of NF1 and for the therapy of myeloid leukemia. In particular, agents that inhibit hyperactive Ras such as farnesyl protein transferase inhibitors are attractive potential therapeutics (38-40). The murine model of JCML that we have developed in the *Nf1* knockout strain in collaboration with the Jacks lab provides an excellent system for testing these and other novel therapies.

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TABLE 1

Parental Transmission of Familal NF1 in Children with Myeloid Disorders*

Sex of Parent with NF1

Sex of Affected Child	Mother	Father
Male	19	8
Female	4	1

* -   Includes the patients in this study; cases described previously as reviewed in Reference (5); and cases in Reference (16).

TABLE 2

NF1 Mutations Detected in Children with Leukemia

Patient	Affected Parent	Diagnosis	LOH at NF1	Mutation Site*	Effect on Neurofibromin
2	Mother	Mo 7	Yes	A to G at nt 6756 + 3 del TTG at nt 6756 +6	truncation at codon 2258
4	Neither	CMML	Yes	C to T at nt 3826	Arg to Stop at codon 1276
5	Father	AML	Yes	4914 del CTCT	truncation at codon 1676
9	Mother	JCML	Yes	C to T at nt 4538	Arg to Stop at codon 1513
10	Neither	JCML	No	2027 ins C	truncation at codon 700
11	Mother	JCML	Yes	5024 del T	truncation at codon 1676
12	Mother	MDS	No	A to G at nt 1642 -8	truncation at codon 1676

Abbreviation: nt = nucleotide

* - The mutations in patients 4, 5, 9, 10, and 11 altered residues in the coding region of NF1. The mutations in 2 and 12 occurred at splice sites and resulted in aberrant mRNAs. The sample from patient 2 showed both a substitution and a 3 bp deletion in the same splice site.

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GRADUATE DEGRESS RESULTING FROM THIS RESEARCH

None